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(54) Title: USE OF LEPTIN TO STIMULATE HEMATOPOIESIS

(57) Abstract

Hematopoietic precursor cell numbers are increased by exposing the cells to leptin. The hematopoietic precursor cells, such as stem cells or progenitor cells of a lin-/c-kit+ fraction, can be separated from mature hematopoietic cells present in a blood product, such as bone marrow, prior to exposure to leptin. The number of hematopoietic stem cells *in vitro* can also be maintained by exposure to leptin, and primitive hematopoietic stem cells can be sustained *in vitro* without substantial proliferation or differentiation. Also, the IL-3 mediated stimulation of hematopoietic cells can be inhibited by treatment with leptin. A composition containing leptin is used to sustain or expand the number of hematopoietic precursor cells. These methods and compositions can be used to support the survival of stem cells during collection and processing, to mobilize stem cells to peripheral circulation, to expand progenitors *in vitro*, to improve bone marrow transplantation, and to increase the efficiency of gene therapy which is targeted to long term repopulating hematopoietic stem cell (LTR-HSC) populations.

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USE OF LEPTIN TO STIMULATE HEMATOPOIESIS

Background of the Invention

I. Leptin, the *ob* gene product

The recently cloned mouse obesity *(ob)* gene encodes an adipose tissue-derived signalling factor for body weight homeostasis. Zhang et al., Nature 372:425-432 (1994). The *ob* gene product was subsequently named "leptin," which means thin in Greek. The leptin protein is proposed to function as part of a signalling pathway from adipose tissue that regulates the size of body fat deposits. *ob/ob* mutant mice are hyperphagic and maintain a body mass eight times of the wild type animal.

The leptin molecule is 167 amino acids long and appears to be well conserved among vertebrates. A cDNA clone encoding human leptin has been isolated and the amino acid sequence shows a high degree of identity between human and mouse. mRNA for leptin is only expressed in white adipose tissue (WAT), and leptin has the properties of a secreted hormone. The fact that leptin is secreted only from fat tissue, taken together with the results from biological studies of the *ob/ob* mice, strongly supports leptin's role as a satiety factor. Administration of recombinant leptin reduces food intake and increases energy expenditure in *ob/ob* mice (Halaas et al., Science 269: 543-546 (1995); Campfield et al., Science 269: 546-549 (1995); Pelleymounter et al., Science 269: 540-543 (1995)), suggesting that it signals to the brain the magnitude of fat stores.

The receptor for leptin has been identified by an expression cloning method (Tartaglia et al., Cell 83: 1263-1271 (1995)). It is a single transmembrane-spanning receptor that appears most related to the gp130 signal-transducing component of the IL-6 receptor, a signal transducer shared by the IL-6, LIF, OM, and IL-11 receptor systems. Strong

evidence indicate that the diabetes (*db*) gene encodes the leptin receptor (Chen et al., Cell 84: 491-495 (1996); Chua et al., Science 271: 994-996 (1996); Lee et al., Nature 379:632-635 (1996)) and that the *db/db* mutation is the result of abnormal splicing of the leptin receptor. At least five alternatively spliced mRNA have been identified in mouse and four in humans, with only one form (the long form) having a signal transducing cytoplasmic domain (Lee et al., supra).

The leptin receptor has a much broader tissue distribution than leptin, and has been detected most strongly in lung and kidney, (Tartaglia et al., supra). With a much more sensitive method (RT-PCR), mRNA for leptin receptor can also be detected in brain, hypothalamus, testis and adipose tissue (Lee et al., supra). A "B219" receptor isolated from yolk sac cell lines, the expression of which was also observed in bone marrow and fractionated bone marrow, has been identified as the leptin receptor. Cioffi et al., Nature Med. 2: 285-589 (1996). Ghilardi et al., Proc. Natl. Acad. Sci. USA 93:6231-6235 (1996), has reported the presence of leptin receptor sequence in bone marrow and many other lymphoid tissues. The receptor appears to be abundant in peripheral and mesenteric lymph nodes.

In several rodent models of obesity, leptin mRNA expression and the level of circulating leptin are increased (Frederich et al., Nature Med. 1: 1311-1314 (1995); Maffei et al., Proc. Natl. Acad. Sci. USA 92: 6957-6960 (1995); and Ogawa et al., J. Clin. Invest. 96: 1647-1652 (1995)). One study reports that a high-fat diet evokes a sustained increase in circulating leptin in both normal and transgenic mice, with leptin levels accurately reflecting the amount of body lipid across a broad range of body fat (Frederich et al., supra). However, despite increased leptin levels, animals fed a high-fat diet became obese without decreasing their caloric intake, suggesting that a high content of dietary fat changes the "set point" for body weight, at least in part by limiting the action of leptin.

Studies have suggested that obese humans and rodents (other than *ob/ob* mice) are not defective in their ability to produce leptin mRNA or protein and generally produce higher levels than lean individuals (Considine et al., J. Clin. Invest. 95: 2986-2988 (1995); Lonnqvist et al., Nature Med. 1: 950-953 (1995); Hamilton et al., Nature Med. 1: 953-956 (1995), Considine et al., N. Engl. J. Med. 334: 292-295 (1996); and Rohner-Jeanrenaud and Jeanrenaud, N. Engl. J. Med. 334: 324-325 (1996)). These data suggest that resistance to normal or elevated levels of leptin may be more important than inadequate leptin production in human obesity.

II. Hematopoietic stem cells (HSC).

The hematopoietic system contains a core of stem cells (HSC) that can expand dramatically on demand and can differentiate to provide all of the myelolymphoid lineages of the circulating population. Till and McCullough, Rad. Res. 14: 213-223 (1961), identified a transplantable cell that could produce millions of descendants (including cells with clone-forming capacity) upon lodgment in the spleens of lethally irradiated mice (Siminovitch et al., J. Cell Comp. Physiol. 64: 23-36 (1964)). This transplantable colony forming cell (CFU-S) is now known to consist of subpopulations with defined capacities to replicate and form specific lineages (Magli et al., Nature 295: 527-529 (1982); Wolf and Priestley, Exp. Hematol. 14: 676-689 (1986)).

In recent years, it has been clear that there must exist a still more "primitive" HSC that does not manifest itself as a CFU-S (the "pre-CFU-S") (Hodgson and Bradley, Nature 281: 381-382 (1979); Pietrzyk et al., Mech. Ageing Dev. 49: 79-86 (1989); Ploechmacher and Brons, Exp. Hematol. 17: 263-266 (1989)). The more primitive this HSC is, the more restricted is its ability to divide under steady-state conditions. A number of attempts have been made to purify these cells using various methods, which have typically included a FACS selection step based either upon cell surface antigenic or lectin determinants or upon differential

affinities for mitochondrial- or DNA-binding vital dyes (Bartelmez et al., Exp. Hematol. 19: 861-862 (1991)).

The cells fractionated by these methods have been studied for proliferative capacity, pluripotency and purity using such measurements as capacity to proliferate and differentiate *in vitro* and/or *in vivo*. The most stringent of these tests is the ability to repopulate all myelolymphoid lineages of a lethally irradiated animal (Metcalf and Moore, Haematopoietic Cells. Frontiers of Biology Series, North Holland, Amsterdam (1971), p. 71. Other approaches have used the transplantation of unfractionated but genetically distinct marrow donor cells, based on either alloenzymes or the random insertion sites of retrovirally transferred genes (Harrison et al., Proc. Natl. Acad. Sci. USA 85: 822-827 (1988); Abkovitz et al., Proc. Natl. Acad. Sci. USA 87:9062-9067 (1990); Dick et al., Cell 42: 7179 (1985); Keller and Snodgrass, J. Exp. Med. 171: 1407-1419 (1990); Jordon and Lemischka, Genes Dev. 4: 220-228 (1990)). These studies, as well as studies which used elutriation methods for marrow cell fractionation (Jones et al., Nature 347: 188-190 (1990)), demonstrated the heterogenous nature of the stem cell compartment with respect to the length of time over which individual clones may function, and the lineage potential of these clones. Distinct HSC subpopulations were demonstrated that ranged from the ability to short-term (weeks to months) repopulate (STR-HSC) to longterm (> 1 year) repopulate (LTR-HSC) lethally irradiated mice (see Bartelmez et al., supra).

Methods have been developed to isolate and separate the subpopulations of LTR-HSC and STR-HSC to an extremely high level of purity (as judged by both *in vivo* and *in vitro* assays). The ability to repopulate transplanted mice and to proliferate clonally *in vitro* have been studied (Wolf et al., Exp. Hematol. 21: 614-622 (1993)). One LTR-HSC is capable of replenishing the marrow of a lethally irradiated mouse. This is accomplished by administering a single LTR-HSC from a male mouse along with STR-HSC from a female mouse to a lethally compromised female mouse. The one LTR-HSC must be accompanied

by STR-HSC to supply the short term needs for blood cells until the LTR-HSC has sufficiently proliferated to support the development of all the blood cell types to allow for long-term survival. The replenishing of the marrow by male donor cells is validated by analysis of the marrow cells for presence of the Y chromosome or the congenic allele Ly 5.1.

In this manner it has been shown that one LTR-HSC can fully repopulate the marrow of a mouse. This indicates significant self renewal of the LTR-HSC which must occur in order for one cell to fully potentiate the whole marrow system. In addition, the presence of LTR-HSC in such a repopulated mouse can be validated by isolation of LTR-HSC from such a repopulated mouse as well as a normal mouse (Jones et al., supra). However, LTR-HSC may be depleted if marrow cells are serially passaged through a number of lethally irradiated mice, indicating that continuous incessant demand for developing cells may deplete the supply of LTR-HSC. These experiments indicate the existence of stem cell renewal and that, under certain circumstances, stem cell renewal can be overwhelmed by factors regulating development.

The process of hematopoiesis is regulated by a group of proteins known as hematopoietic growth factors including colony stimulating factors and interleukins. Growth and development of hematopoietic cells is under strict regulation by these factors requiring constant presence of factor(s) for continued growth and differentiation. Different levels and/or mixtures of factors may account for developmental determination at points in stem cell differentiation and maturation of progeny. The effects of these factors are mediated through cell surface proteins known as hematopoietic receptors. There are factors such as stem cell factor (SCF), granulocyte macrophage colony stimulating factor (GM-CSF), and macrophage colony stimulating factor (M-CSF) which affect early, middle, and late stage in vitro development respectively.

By analogy to the regulation of other hematopoietic cells, self-renewal of stem cells is thought to be regulated

by factor(s) mediating their effect through cell surface receptors regulating this process. To date the responsible factor(s) is unknown.

The primary control mechanisms by which the hematopoietic hierarchy is maintained are unknown. For example, vertebrates maintain what appears to be a very finely regulated production of all blood cell types that is generated by a hierarchy of marrow cells that progressively give rise to daughter cells with less proliferative and differentiation potential. This hierarchy may be maintained at all levels by varying degrees of self-replication within a "compartment," and also by differentiation of cells into and out of a compartment. It would be useful to identify self-replication factors (SRFs) in these compartments.

Most hematopoietic growth factors act as potent mediators of differentiation, and as such are classic differentiation factors (DFs). However, the most primitive cells of the stem cell compartment are unique in that there are no cells that can differentiate into this compartment. A differentiation event occurs within the stem cell compartment when LTR-HSC divide and generate STR-HSC which appear to have limited clonal lifespans (see, Bartelmez et al., Exp Hematol 2:861-862 (1991)). However, the LTR-HSC appear to have an unlimited lifespan which could be accounted for through an asymmetrical division (one daughter cell is a replicate of the mother cell, the other enters the hematopoietic pool of cells with limited clonal lifespans). However, only a symmetrical division of self replication would lead to clonal expansion. Evidence that clonal expansion occurs is provided by in vivo data obtained using retrovirally marked, serially transplanted marrow, where the expansion of individual HSC clones is clearly observed (Dick et al., Cell 42:71 (1985); Keller and Snodgrass, J. Exp. Med. 171: 1407 (1990); Jordan and Lemischka, Genes Dev. 4: 220 (1990)). The mechanism that provides this expansion is unknown, but it may be intrinsic or extrinsic (Wolpert, J. Cell Sci. (Suppl) 10:1-9 (1988); Ogawa et al., Blood 81: 2844-2853 (1993)). Transforming growth

factor-beta-1 (TGF-ß1) has been identified as an extrinsic and intrinsic (Sitnicka et al., Blood 88: 82-88 (1996)) mediator of self-renewal of LTR-HSC.

In culture, in the presence of a known growth factor or combination of factors, LTR-HSC do not undergo self renewal. Culturing stem cells in the presence of cell condition media or on feeder cells has also not been reported to stimulate self renewal of LTR-HSC in culture (Knobel et al., Exp. Hematol. 22: 1227-1235 (1994); van der Loo and Ploemacher, Blood 85: 2598-2606 (1995). To confirm the existence of such a factor, the cDNA encoding this activity must be isolated.

There remains a need in the art for a method of culturing hematopoietic cells which results in expansion of the number of hematopoietic precursor cells and enhances the yield and recovery of the precursor cells without compromising viability. Quite surprisingly, the present invention addresses this and other related needs.

Summary of the Invention

The present invention provides, in one aspect, methods for increasing the number of hematopoietic precursor cells by exposing the precursor cells to leptin, thereby increasing the number of precursor cells relative to the number of cells initially present. The exposure of the cells can occur either in vitro or in vivo. The hematopoietic precursor cells can be separated from mature hematopoietic cells present in a blood product, such as bone marrow, umbilical cord blood, or peripheral blood, prior to exposing the hematopoietic precursor cells to leptin. The hematopoietic precursor cells are stem cells or progenitor cells. Typically the hematopoietic precursor cells which are exposed to the leptin are lin- cells, and more preferably are a lin-/c-kit+ fraction thereof, and include a lin-/Rh low/c-

kit+ and lin-/Rh high/c-kit+ cellular fraction. Although cells from a variety of mammals can be employed in these methods, when the cells are intended for administration to a human they will typically be human in origin. When the cells are exposed to the leptin in vitro in a culture medium, typically at least one exogenously added growth factor, such as G-CSF, GM-CSF, IL-1, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 or TGF-β, will be included.

In another embodiment the invention provides a method for maintaining the number of hematopoietic stem cells in vitro, comprising exposing the stem cells to leptin and culturing the cells. The number of stem cells relative to the number of cells initially present is thereby maintained. The hematopoietic stem cells can be separated from mature hematopoietic cells present in a blood product, such as marrow or umbilical blood, prior to exposing the stem cells to leptin. The hematopoietic stem cells are typically lin-/c-kit+ cells or a lin-/Rh low/c-kit+ or lin-/Rh high/c-kit+ fraction thereof.

In yet another aspect the invention provides a method for sustaining primitive hematopoietic stem cells in vitro without substantial proliferation or differentiation. In this method the primitive hematopoietic stem cells are exposed to leptin and then cultured, and the number of primitive stem cells relative to the number of cells initially present is sustained, e.g., for at least five to seven days. Typically the primitive hematopoietic stem cells are lin-/Rh low/Ho low/c-kit+ or lin-/c-kit+, and may be separated from mature hematopoietic cells present in a blood product prior to exposing the primitive hematopoietic stem cells to leptin. The primitive hematopoietic stem cells can be subsequently stimulated to proliferate and differentiate.

In another embodiment the invention provides a method for inhibiting the proliferation of hematopoietic cells that have been stimulated by IL-3. IL-3 stimulated hematopoietic cells are exposed to leptin, thereby inhibiting

the proliferation of cells induced by IL-3. The hematopoietic cells can be in fractionated or unfractionated bone marrow.

In yet another embodiment the invention provides a method for increasing the number of hematopoietic cells in a mammalian host. According to this method, the hematopoietic precursor cells of the host are exposed to leptin, either in vitro or in vivo, thereby increasing the number of precursor cells relative to the number of cells initially present. When in vitro, the hematopoietic precursor cells can be separated from mature hematopoietic cells present in the blood product such as bone marrow prior to exposing the cells to leptin. Typically the hematopoietic precursor cells, of a lin-fraction, such as lin-/c-kit+, are obtained from the host prior to initiation of cytotoxic therapy.

A composition for sustaining or expanding the number of hematopoietic precursor cells is also provided. The composition comprises a nutrient medium and leptin. Typically the composition further comprises at least one exogenously supplied growth factor, such as G-CSF, GM-CSF, SCF, IL-1, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 or TGF-β.

Brief Description of The Figures

Fig. 1 shows the detection of leptin receptor mRNA expressed in bone marrow cells and hypothalamus, where 1 μg, 0.1 μg and 0.01 μg of DNA from cDNA library prepared from RNA of hypothalamus (lanes a, d, g, respectively), bone marrow (lanes b, e, h, respectively) and ALVA 31 prostrate tumor cells (lanes c, f, i, respectively) were used as template for PCR amplification using primers which recognize a common sequence found in known forms of leptin receptor. The amplified DNA was fractionated by 1 % agarose gel electrophoresis, stained with ethidium bromide, excited with UV light, and photographed. The 100 bp ladder (Pharmacia) was used as marker DNA.

Fig. 2 shows the detection of multiple forms of leptin receptor mRNA expressed in bone marrow, where 1 μg of DNA from the bone marrow cDNA library was used for PCR amplification with primers specific for each alternatively spliced forms of leptin receptor. Lanes a, b, c, d, and e, show Ra, Rb, Rc, Rd and Re forms of leptin cDNA, respectively, and a 100 bp ladder was used as marker DNA.

Detailed Description Of The Specific Embodiments

The present invention provides compositions and methods for maintaining and increasing the number of hematopoietic precursor cells in vitro and in vivo. Specifically, the present invention provides methods to sustain the growth and differentiation of hematopoietic precursor cells in vivo or ex vivo by exposing the cells to leptin or leptin-like proteins or analogs which are capable of stimulating leptin receptor-mediated activity in these cells. Leptin and analogs thereof can also be used in the present invention to sustain self replication of primitive hematopoietic stem cells in vitro, to inhibit IL-3 stimulated differentiation of hematopoietic cells, and to enrich for hematopoietic cells expressing leptin receptor in a mixed population of hematopoietic cells. In another aspect, leptin can be used synergistically with other exogenously added hematopoietic growth factors to support and expand hematopoietic stem and progenitor cells. The ability to sustain and expand hematopoietic precursor cells, derived from the bone marrow, peripheral blood, or umbilical cord blood of a patient or donor, counters some of the disadvantages of immunosuppressive or immunodestructive therapies which are often used in the treatment of cancer and other life-threatening diseases. For example, cultured hematopoietic cells can be used as an important source of proliferating cells to reconstitute a patient's blood-clotting and

infection-fighting functions subsequent to therapy. In addition, the ability to expand hematopoietic precursor cells and their precursors in vitro relieves dependence on bone marrow aspiration or multiple aphereses as the only means of obtaining sufficient cells for transplantation.

For in vitro use of leptin in the present invention, hematopoietic precursor cells are typically separated from a blood product, such as bone marrow, peripheral blood, or umbilical cord blood of a patient or donor, fetal peripheral blood and other sources. As discussed in more detail below; such separation may be performed, for example, by depleting the blood product of cells that are committed to specific lineages based on their expression of one or more antigens which are present on substantially all lineage committed cells but are substantially absent from the lineage committed precursor cells. This lin- fraction is enriched for hematopoietic progenitor cells. The lin- cells can be further fractionated, e.g., based on their expression of c-kit on the cell surface into c-kit+ and c-kit- fractions, as described in more detail below, and fractionated even further based again of differential expression of cell surface markers associated with a desired subpopulation. The separated hematopoietic precursor cells may be stored frozen and thawed at a later date for inoculation into a suitable vessel containing a culture medium comprising a nutritive medium and leptin, optionally supplemented with a source of growth factors and, optionally, human or other animal plasma or serum. Alternatively, the separated cells may be inoculated directly into culture without first freezing. In both cases the resultant cell suspension is cultured in the presence of leptin under conditions and for a time sufficient to increase the number of hematopoietic precursor cells relative to the number of such cells present initially in the blood product. The cells may then be separated by any of a variety of methods, such as centrifugation or filtration, from the medium in which they have been cultured, and may be washed one or more times with fresh medium or buffer. Optionally,

the cells may be re-separated into lin- and lin+ fractions prior to resuspension to a desired concentration in a medium or buffer suitable for infusion. The cells may then be infused into a patient or stored frozen for infusion at a later date.

Surprisingly, separated precursor cells, such as lin- cells and lin-/c-kit+ cells, will expand in number when cultured in the presence of medium containing leptin, enabling clinically practicable expansion and recovery of hematopoietic precursor cells. By working with separated precursor cells, the volumes of cells and culture fluids which must be handled are reduced to more manageable numbers. Further, a high degree of expansion can be achieved when one starts with separated lin- and lin-/c-kit+ cells, rather than with an unseparated blood product. This is believed to be due to the removal of cells otherwise present in the blood product, which inhibit expansion of the precursor cells. Under the conditions employed in the methods of this invention, cell recovery is greatly facilitated and viability is preserved. Most importantly, the yield of hematopoietic precursor cells, capable of mediating both long-term and short-term hematopoietic recovery in a myelosuppressed or myeloablated host, is increased. The ability to sustain or expand hematopoietic precursor cells in vitro or in vivo by the compositions and methods of the present invention is expected to have tremendously important consequences for disease treatments which are inherently myelosuppressive or myeloablative, such as in cancer chemotherapy.

Within the context of the present invention, the lin- hematopoietic precursor (non-committed) cells include those which do not express antigens associated with neutrophils and activated macrophages (e.g., 7/4), B and pre-B lymphocytes (e.g., B220), erythrocytes (YW 25.12.7), neutrophils and macrophages (e.g., GR-1), T cells (Lyt-2), T-helper cells (e.g., L3T4), nucleated red blood cells (e.g., Ter 119) and include primitive, totipotent stem cells as well as progenitor cells. Lin- cells/c-kit+ cells are those lin-

cells which express c-kit antigen, as can be conveniently determined with an antibody to c-kit. Even further fractionation of lin- or lin-/c-kit+ cells based on amount of rhodamine 123 fluorescence can be used to provide fractions of lin-/Rh low or lin-/Rh low/c-kit+ cells containing primitive hematopoietic cells, whereas cells of the Rh high fraction are less primitive. The level of expression of the cell surface antigens will vary from one cell type to another. Consequently, a cell is operationally defined as lin-, c-kit+, or Rh low, if it expresses or does not express sufficient antigen to be detected by a given method of assay. For example, c-kit+ cells can be identified by flow microfluorimetry using a fluorescence-activated cell sorter (FACS), by immunofluorescence or immunoperoxidase staining using a fluorescence or light microscope, by radioimmunoassay, or by immunoaffinity chromatography, among numerous other methods which will be readily apparent to one skilled in the art (see, for example, Lansdorp and Thomas (in Bone Marrow Processing and Purging, A.P. Gee (ed.), Boca Raton: CRC Press (1991) pg. 351). Hematopoietic precursor cells can also be detected by various colony-forming assays, such as CFU-GM and CFU-S assays (see, e.g., Till and McCullough, supra; Bradley and Metcalf, Aust. J. Exp. Biol. Med. Sci. 44: 287 (1966)).

Hematopoietic precursor cells, including lin- and lin-/c-kit+ cells, may be obtained from any of a variety of blood products, including bone marrow, peripheral blood, umbilical cord blood, fetal liver, and spleen. Bone marrow is a particularly rich source of precursor cells (1-2% of marrow), but alternate sources may be preferable because of the discomfort associated with bone marrow aspiration. Bone marrow is typically aspirated from the iliac crest, but may be obtained from other sites (such as the sternum or vertebral bodies) if necessitated by prior or concurrent disease or therapy. Peripheral blood contains fewer precursor cells (typically < 1% of peripheral blood mononuclear cells), but is generally easier to obtain than bone marrow. The number of

precursor cells circulating in peripheral blood can be increased by prior exposure of the donor to certain growth factors, such as, for example, G-CSF or SCF (*kit* ligand (KL)), and/or certain drugs, such as, for example, cyclophosphamide or prednisone. Peripheral blood collected from patients or donors who have been pretreated to increase the number of circulating hematopoietic precursor cells is referred to as having been "mobilized." According to one aspect of the invention, leptin can be used to mobilize hematopoietic stem cells from the marrow to the peripheral blood for collection. Depending upon the volume which is desired, blood may be obtained by venipuncture or by one or more aphereses. Precursor cells can also be obtained from umbilical cord blood at the time of delivery, either by simple gravity-induced drainage or manual expression.

Separation of precursor cells from more mature cells can be accomplished by any of a variety of methods known to those skilled in the art, including immunoaffinity chromatography (Basch et al., J. Immunol. Methods 56:269 (1983)), fluorescence-activated cell sorting, panning (Wysocki and Sato, Proc. Natl. Acad. Sci. USA 15: 2844 (1978)), magnetic-activated cell sorting (Miltenyi et al., Cytometry 11: 231 (1990)), and cytolysis. According to the present invention, leptin is used to support survival of stem cells during the collection and processing of whole or fractionated marrow. Generally, separation of a heterogeneous population of cells, such as in a bone marrow aspirate or a peripheral blood specimen or apheresis product, into target (e.g., CD34+ or lin-) and non-target (e.g., CD34- or lin+) fractions is rarely complete. For the purposes of the present invention, separation is considered to have been accomplished if the target fraction is comprised of at least about 20% precursor cells, more often about 50% precursor cells, and preferably about 70% precursor cells. In addition, it may be desirable to keep the total numbers of mature hematopoietic cells, such as platelets, granulocytes, and red cells, as low as possible in order to prevent clumping and the release of degradative

enzymes which can adversely affect the recovery and viability of engrafting cells, especially after freezing and thawing. More specifically, it may be desirable that the target fraction be comprised of less than about 5% platelets, 50% granulocytes, and 10% red cells and, preferably, less than about 1% platelets, 25% granulocytes, and 1% red cells.

Precursor cells may be positively selected or negatively selected. By positive selection is meant the capture of cells by some means, usually immunological, on the basis of their expression of a specific characteristic or set of characteristics (usually an antigen(s) expressed at the cell surface). For example, CD34+ or c-kit+ cells can be positively selected by any of the above methods (except cytolysis, which would result in destruction of the desired cells) on the basis of their expression of the c-kit antigen utilizing an anti-c-kit antibody, such as anti-human monoclonal antibody YB5.B8 (available from Pharmingen, Inc.), or anti-CD34 monoclonal antibodies which are also commercially available, e.g., monoclonal antibodies 12.8, My-10, or 8G12 (Becton Dickinson Co., Mountain View, CA).

Negative selection means the exclusion or depletion of cells by some means, usually immunological, on the basis of their lack of expression of a specific characteristic or set of characteristics (again, usually a surface antigen). For example, lin- cells can be negatively selected by any of the above methods on the basis of their lack of expression of lineage-defining antigens, such as CD19 (for B lymphocytes), CD3 (for T lymphocytes), CD56 (for NK cells), CD4 (T-helper cells), CD8 (cytotoxic T cells), CD20 (macrophages), CD11b (monocytes, granulocytes, neutrophils, macrophages, NK cells, and activated lymphocytes), CD33 (monocytes, activated T cells, myeloid progenitors), glycophorin A (red blood cells and erythroid precursor cells), etc., utilizing antibodies to the above-mentioned and other lineage-defining antigens. By using a cocktail or mixture of monoclonal antibodies directed to red cell, platelet, granulocyte, lymphocyte and/or tumor cell antigens, it is possible to leave behind a population of

cells which is highly enriched for lin- cells. Numerous monoclonal and polyclonal antibodies suitable for this purpose are known in the art (see Leukocyte Typing IV, Knopp et al. (eds.), Oxford UP, 1989) and are commercially available from a wide variety of sources (for example, Becton Dickinson Co., Mountain View, CA; Coulter Immunology, Hialeah, FL; Ortho Diagnostics, Raritan, NJ, etc.).

Alternatively, precursor cells can be separated from mature cells by a combination of negative and positive selection techniques. A preferred combination of negative and positive selection techniques is comprised of a first selection for lin- cells utilizing a mixture of anti-lin+ antibodies as described above, followed by a second selection for c-kit+/lin- cells, using an anti-c-kit antibody to a determinant on the c-kit antigen. Antibodies to determinants on the c-kit molecules are well-known in the literature (see, e.g., Ashman et al., J. Cell. Physiol. 158: 545 (1994)) and are available from a variety of sources, including those mentioned above. The advantage of this or other dual selection strategies is that the volume of cells which is placed into culture is smaller, more manageable, and more susceptible to stimulation and expansion by leptin.

During the process of leptin-stimulated expansion according to the invention, the precursor cells are periodically (e.g., every 4-7 days) separated from more mature cells. Briefly, mature cells (which include not only terminally differentiated blood cells, but cells of an intermediate lineage) may inhibit the expansion and differentiation of precursor cells via a feedback control mechanism. Removal of more mature cells from a culture thus permits expansion of the precursor cells to many times their original numbers. Various methods may be used to periodically separate precursor from mature cells, for example, cells can be separated on an affinity column, incubated in a selected medium, and then subsequently reseparated in order to separate the precursor cells from the newly differentiated mature cells. Examples of methods and devices for selecting

precursor cells, such as CD34+, or lin-/c-kit+ cells, are described in U.S. Patent Nos. 5,215,927, and 5,225,353, each of which is incorporated herein by reference.

Subsequent to separation, precursor cells are inoculated into a culture medium comprised of a nutritive medium, any number of which, such as RPMI, TC 199, Ex Vivo-10, or Iscove's DMEM, along with a source of growth factors, will be apparent to one skilled in the art. Proliferation and differentiation of precursor cells may be enhanced by the addition of various components to the medium, including a source of plasma or serum. Among sources of plasma or serum are fetal bovine and human. The amount of plasma or serum which is used will vary, but is usually between about 1 and 50% (by volume) of the medium in which the cells are grown, and more often between about 1 and 25%.

According to one aspect of the present invention, separated hematopoietic precursor cells are cultured in a nutritive medium and leptin. The leptin is prepared as generally described in, e.g., Friedman et al., International PCT publication WO 96/05309, incorporated herein by reference. WO 96/05309 describes the preparation of isolated and purified leptin (alternatively described as OB polypeptide), as well as nucleic acids encoding leptin and the amino acid sequences thereof, including human leptin polypeptides, and peptide fragments, derivatives and analogs. The leptin polypeptides for use in the methods of the present invention can be prepared by recombinant or synthetic means, as described in WO 96/05309. Other leptin receptor recognition molecules can also be used in the present invention. For example, leptin analogs and mimetics, including small molecule analogs, can be identified as leptin receptor agonists or antagonists as described in WO 96/05309. A leptin receptor has been described in Tartaglia et al., Cell 83:1263-1271 (1995), incorporated herein by reference. The mimetics or analogs will typically have a biological activity of the leptin polypeptide, including but not limited to specific binding to a leptin receptor or antibody specific for a leptin molecule, or other

recognition molecule; activation of signal transduction pathways; and/or induction (or inhibition by antagonists) of physiological effects mediated by the native leptin polypeptide in vivo or in vitro. It is understood that reference herein to leptin includes molecules such as leptin mimetics and the like which have the same or similar effect as leptin as described herein, e.g., maintaining or expanding hematopoietic precursor cells, sustaining self-replication of primitive stem cells, inhibiting differentiation of hematopoietic precursor cells caused by IL-3, and improving the recovery of patients from radiation or chemotherapy.

The leptin can be used in the present invention as a purified preparation or as a component of a composition, including a mixture with other hematopoietic growth factors. For example, among growth factors which may be employed in the medium are interleukins (IL) 1-15, erythropoietin (US Patent No. 4,703,008, incorporated herein by reference), stem cell factor (SCF, also known as mast cell growth factor and c-kit ligand), granulocyte colony stimulating factor (G-CSF), granulocyte, macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), transforming growth factor beta (TGF beta), tumor necrosis factor alpha (TNF alpha), thrombopoietin (TPO), the interferons (IFN alpha, beta, or gamma), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factors (IGF-1 and IGF-2), megakaryocyte promoting ligand (MPL) and SLK-2, etc. Growth factors are commercially available, for example, from R & D Systems, Inc. (Minneapolis, MN), Amgen, Inc. (Thousand Oaks, CA), Immunex Corp. (Seattle, WA), Genetics Institute (Cambridge, MA), and Genentech, Inc. (S. San Francisco, CA), among other sources. Particularly preferred are combinations of growth factors, especially the combination of IL-3, G-CSF, and SCF. In general, the above-mentioned growth factors are purified or partially purified before they are added to the culture medium. Usually, they will be produced by recombinant DNA methods, but they may also be purified by standard biochemical techniques

from conditioned media. Non-naturally occurring growth factors can also be produced by recombinant DNA methods, and it will be evident to those skilled in the art that fusion proteins, combining multiple growth factor activities, can be readily constructed, for example, fusion proteins combining SCF activity with that of other growth factors such as IL-1, IL-6, G-CSF, and/or GM-CSF. The amount of each growth factor to be used is determined empirically and will vary depending on the purity and method of production of the factors. Generally, concentrations between 0.5 and 100 ng/ml are sufficient, more often between 0.5 and 50 ng/ml. Where more than one growth factor is used, the optimum amount of each factor should be determined in combination with the other factors to be used. This is because some growth factors can modulate the activity of other growth factors, necessitating that they be used sequentially rather than simultaneously, while in other instances, growth factors may act synergistically. Still other growth factors may enhance proliferation or differentiation along one pathway, while suppressing another pathway of interest. For example, as shown herein leptin suppresses the stimulation of proliferation that is stimulated by IL-3. Thus, leptin can be used in this situation to suppress the amplification of certain cell types, e.g., those which are IL-3 responsive, while permitting the amplification of others.

The medium may also be prepared with or without a source of serum or plasma. If used, the serum or plasma may be of human or other animal origin. Particularly preferred for cultivation of human cell is human autologous plasma or human AB^- plasma. The amount of plasma or serum which is used will vary, but is usually between about 1 and 50% (by volume) of the medium in which the cells are grown, and more often between about 1 and 25%.

Separated precursor cells may be cultured in any vessel which is capable of being sterilized, is adapted or adaptable to gas exchange with the atmosphere, and is constructed of a material which is non-toxic to cells. A

variety of vessels suitable for this purpose are well-known in the art, including stirring flasks (Corning, Inc., Corning, NY), stirred tank reactors (Verax, Lebanon, NH), airlift reactors, suspension cell retention reactors, cell adsorption reactors, and cell entrapment reactors, petri dishes, multiwell plates, flasks, bags and hollow fiber devices. If agitation is desired, it can be attained by any of a variety of means, including stirring, shaking, airlift, or end-over-end rotation. In addition to maintaining the culture in suspension by agitating the medium (as by stirring or airlift), the culture can also be maintained in suspension by matching the density of the culture medium to the density of the cells or microcarrier beads.

This invention provides methods of using leptin and pharmaceutical compositions comprising leptin for stimulating hematopoiesis in mammals, including humans. These compositions and methods are useful in promoting both long-term and short-term hematopoietic recovery and treating side effects of in a myelosuppressed or myeloablated host. Myelosuppression or myeloablation may occur as an isolated disorder, secondary to another disorder, or as a side effect resulting from a specific therapeutic regimen. The leptin can be used alone or in conjunction with other treatments, e.g., G-CSF. The leptin is typically administered in the form of a pharmacologically effective amount and a pharmaceutically acceptable carrier. A pharmacologically effective amount is, in this case, an amount effective to promote the proliferation of hematopoietic cells, e.g., stem or progenitor cells, or increase the number of stem cells in the circulating blood.

The form, amounts and timing of administration generally are a matter for determination by the practitioner. For the agents of this invention, systemic administration in a liquid carrier by injection is preferred. In one embodiment, the pharmaceutical composition is delivered as a unit dosage form. For example, unit dosage forms for systemic administration include unit doses of injectable solutions. These compositions are useful in the therapeutic methods of

this invention. Therapeutic doses generally will be in the range of 0.1 μg/kg to 1.0 mg/kg of subject body weight per day, preferably 1.0 μg/kg to 100 μg/kg body weight per day, with the exact dose determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, the subject's traits, etc. Determination of dose is within the level of ordinary skill in the art. The protein may be administered for acute treatment, over one week or less, often over a period of one to three days or may be used in chronic treatment, over several months or years.

For use of the present invention in bone marrow transplantation (the transplantation of hematopoietic cells into the bone marrow of a subject), methods are provided for transplanting hematopoietic cells maintained and/or expanded with leptin into a subject. Bone marrow transplantation is useful for replacing hematopoietic cells in subjects whose hematopoietic cells have been damaged or destroyed by, for example, disease, cancer therapy or other exposure to radiation. In one version of the method, bone marrow or peripheral blood progenitor (PBPC) cells are removed from a patient or a suitable donor prior to the patient's chemotherapy or radiation exposure and treated with leptin, optionally in combination with one or more other cytokines, as described above. The treated marrow or PBPCs are then returned to the patient after chemotherapy to speed the recovery of the marrow. Prior to chemotherapy treatment, marrow can be stimulated with stem cell factor (SCF) or G-CSF to release early progenitor cells into peripheral circulation. These progenitors can be collected and concentrated from peripheral blood and then treated in culture with leptin, optionally in combination with one or more other cytokines, including but not limited to SCF, G-CSF, GM-CSF, IL-6 or IL-11, to differentiate and proliferate into high-density megakaryocyte cultures, which can then be returned to the patient following high-dose chemotherapy.

In another aspect the present invention can be used in methods of hematopoietic stem cell transplantation. Procedures for obtaining hematopoietic stem cells, readministering them and repopulating marrow are widely used. To inhibit loss of "stemness" in vitro and to facilitate survival during collection and processing, the cells are exposed to effective concentrations of leptin during these stages, including during cultivation. This treatment enhances repopulation of the marrow by stem cells upon subsequent readministration to the patient.

Leptin can also be used in methods of gene therapy where hematopoietic cells are targeted for introduction of one or more genes encoding expression product(s). Hematopoietic stem cells are often targets of gene therapy methods, but in the past it has been difficult to manipulate the stem cells in vitro without losing the stemness of the long term repopulating stem cells (LTR-HSC). To maintain long term expression of a foreign gene product by gene therapy methods, the LTR-HSC must be altered in such a way that stemness has been maintained. According to this aspect of the invention, exposure of the LTR-HSC to leptin during collection and manipulation in vitro promotes survival and expansion of this cell population, thereby enhancing the long term effectiveness of the gene therapy procedure. Suitable viral vectors include retroviral vectors (see Miller, Curr. Top. Microbiol. Immunol. 158: 1-24 (1992); Salmons and Gunzburg, Human Gene Therapy 4: 129-141 (1993); Miller et al., Methods in Enzymology 217: 581-599, (1994)) and adeno-associated vectors (reviewed in Carter, Curr. Opinion Biotech. 3: 533-539 (1992); Muzcyzka, Curr. Top. Microbiol. Immunol. 158: 97-129 (1992)). Other viral vectors that may be used within the methods include adenoviral vectors, herpes viral vectors and Sindbis viral vectors, as generally described in, e.g., Jolly, Cancer Gene Therapy 1:51-64 (1994); Latchman, Molec. Biotechnol. 2:179-195 (1994); and Johanning et al., Nucl. Acids Res. 23:1495-1501 (1995), each incorporated herein by reference. The choice of vector will rely in part on the requirement for cellular replication, the

disease state that is being treated and the size of the gene to be transferred.

In some instances, it may be useful to transfect hematopoietic cells or other cells with an expression vector capable of expressing leptin and introducing such cells into a subject. Such methods are useful for increasing the amount of leptin activity in the subject. Increasing leptin activity would be useful in the treatment of, for example, myelosuppression or the like. In one embodiment, the genes are introduced into cells of the individual *in vivo* by means of expression vectors. In another embodiment, the genes are introduced into cells *ex vivo*, and transfected cells that express and secrete leptin are administered to the subject.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE 1

Expression of Leptin Receptor in Bone Marrow Cells

PCR primers were initially designed according to the mouse and human leptin receptor sequences to detect receptor cDNA sequence in a hypothalamic cDNA library.

DNA from the cDNA library was first denatured by alkaline treatment, diluted, and subjected to PCR amplification with leptin receptor specific sequences. For the initial experiment, a cDNA library constructed from bone marrow tissue RNA was included as a control.

For isolation of RNA from tissue or cultured cells, total RNA was extracted from mouse bone marrow tissue by the method of Chomczynski and Sacchi, Analyt. Biochem. 162: 156-169 (1987) and using RNAzol solution supplied by the manufacturer (Tel-Test, Inc. Friendswood, Tx). Tissues were deposited directly into appropriate volume of RNAzol solution. Extraction of RNA followed the procedures recommended by the manufacturer with slight modifications. Two additional

phenol-chloroform extractions were routinely performed on the RNA isolated by the RNAzol method to ensure the purity of RNA.

Primers for PCR were designed based on leptin receptor sequence using the GeneWorks program (IntelliGenetics). Primers were purchased from Gibco/BRL. The PCR primer set sequence was designed to amplify sequence from amino acid #8 through amino acid #248 at the 5' end of the coding region of leptin receptor, and to detect all alternatively spliced forms of leptin receptor mRNA. The pair of primers had the following sequence: forward primer, 5' TGTGGTTTTGTTACAYTGGG 3' [Seq ID No: 1]; reverse primer, 5' TGTGAYTTCCATATGCAATCC 3' [Seq ID No: 2]. PCR was performed with standard method recommended by the manufacturer on a Gene Amp 2400 system (Perkin-Elmer). Taq polymerase and reagents were purchased from Perkin-Elmer. PCR was generally done with 25-30 cycles of amplification. PCR product was analyzed on a 1% agarose gel.

For reverse transcription-PCR (RT-PCR), a standard protocol was followed as described in Rao et al., FEBS Lett. 263: 18-22 (1990), incorporated herein by reference. Total RNA was first reverse transcribed with either specific primer or random primer by SuperScript II (GIBCO-BRL). The RT reaction followed recommendations of the manufacturer. A portion of, or diluted cDNA was used for PCR depending on the abundance of the mRNA. PCR amplification was as described above, and RT-PCR product was analyzed on an appropriate percentage of agarose gel.

The results obtained with the hypothalamic cDNA library and cDNA libraries constructed from bone marrow RNA and from ALVA31 prostate tumor cell RNA are shown in Fig. 1. A 720 bp band was detected from 1 μg and 0.1 μg of hypothalamic cDNA library DNA (Fig. 1, lanes a and d). Surprisingly, the 720 bp band was also amplified from DNA of the bone marrow library (Fig. 1, lanes b, e, and h). The intensity of the 720 bp DNA band amplified from the bone marrow library was greater than from the hypothalamic library. The 720 bp band could be detected from as little as 0.01 μg of

the total bone marrow library DNA. The 720 bp band was isolated from agarose gel and partially sequenced to confirm its identity as the leptin receptor sequence.

The primer set used for the initial experiments amplified all forms of leptin receptor. Alternatively spliced forms of leptin receptor mRNA are not revealed by this analysis. There are at least five alternatively spliced forms of mRNA for leptin receptor, and only one form (form b) contains a cytoplasmic signal transducing domain. Which alternatively spliced form of leptin receptor mRNA is expressed in bone marrow tissue was determined. Specific PCR primer sets have been used to differentially detect all five forms of alternatively spliced mRNA (Lee et al., Nature 379: 632-635 (1996)). PCR was performed with 1 ug of DNA from total bone marrow cDNA library using the following primers: for Ra, forward: 5' ACACTGTTAATTTCACACCAGAG 3' [Seq ID No: 3], reverse: 5' AGTCATTCAAACCATTAGTTTAGG 3' [Seq ID No: 4]; for Rb, forward: 5' ACACTGTTAATTTCACACCAGAG 3' [Seq ID No: 5], reverse: 5' TGGATAAACCCTTGCTCTTCA 3' [Seq ID No: 6]; for Rc, forward: 5' ACACTGTTAATTTCACACCAGAG 3' [Seq ID No: 7], reverse: 5' TGAACACAACAACATAAAGCCC 3' [Seq ID No: 8]; for Rd, forward: 5' ACACTGTTAATTTCACACCAGAG 3' [Seq ID No: 9], reverse: 5' AGGCTCCCTCAGGGCCAC 3' [Seq ID No: 10]; for Re, forward: 5' TGTTATATCTGGTTATTGAATGG 3' [Seq ID No: 11], reverse: 5' CATTAAATGATTTATTATCAGAATTGC 3' [Seq ID No: 12]. The results are shown in Fig. 2. Except for form d (lane d), which can not be determined due to high background, all other forms can be detected in the cDNA library (forms a, b, c, and e in lanes a, b, c, and e respectively). Form b, the long form is also present in the cDNA library, however, the intensity of PCR product for form b (lane b) appears to be the least of all forms.

As bone marrow cells are a heterogeneous set of cells, one goal was to identify cell types within the population that express the leptin receptor mRNA by using the RT-PCR method, based on the assumption that cells expressing the leptin receptor will be able to respond to leptin. Cell

lines screened included WEHI-3 cells, 32D-J558 cells, EML cells and MPRO cells. As a result of the screening, only WEHI-3 cells were found to express the receptor sequence. WEHl-3 is a mouse myelomonocytic leukemia cell line. WEHI-3 cellular RNA was then used to determine the sensitivity of detection by the RT-PCR method and to determine whether the leptin receptor mRNA was the long or short form. The WEHI-3 RNA was also used to standardize and improve the sensitivity of detection by RT-PCR method.

A bone marrow library was constructed with RNA isolated from bone marrow of an animal treated with 5-Fluorouracil (5-FU) to enrich for hematopoietic stem cell population and to determine whether 5-FU would enrich for the cells expressing leptin receptor mRNA. 5-FU treatment ablates dividing cells and therefore enriches for precursor and mature cells of the bone marrow (Bartelmez and Stanley, J. Cell. Physiol. 122:370-378 (1985)). Male and female F_1 hybrid mice from the cross C57B1/6 X DBA/2 were used. Three-to-six-month old mice came from a NIA-derived breeding colony maintained under strict specific pathogen-free conditions and routinely tested and confirmed to be free of all known mouse pathogens. Mice of about 20 g were either treated with a single injection of 3 μg 5-FU or not treated, and bone marrow cells were harvested 3 days after treatment.

To harvest bone marrow cells, mice were sacrificed, femurs and tibias were removed aseptically and repetitively flushed of marrow with phosphate buffered saline (PBS), 2% fetal bovine serum (FBS) (Biocel, Rancho Dominguez, CA). This solution was used as collection medium and for cell resuspension through lineage depletion steps described below. The low density bone marrow cells were isolated on a gradient by layering 5 ml aliquots of 10^7 cells/ml over 4.0 ml Nycodenz (1.080 g/ml) (Nycomed, Oslo, Norway). Cells were centrifuged 20 min at 400 x g, collected from the interface and washed twice with collection medium at 4°C.

RNA was recovered from the marrow cells of 5-FU treated or untreated mice, quantitated and equal amount of RNA

was subject to RT-PCR. After reverse transcription (RT), total cDNA was then diluted to compare the sensitivity of detection from cell population of either 5-FU treated or untreated animals. The results showed no dramatic difference between samples from animals treated with 5-FU compared to samples from animals not treated with 5-FU. These results indicate that expression of leptin receptor might be enriched in the stem cell or stromal cell populations, cells which are relatively non-proliferating and thus less affected by 5-FU treatment. With identical conditions leptin receptor signal can be detected from 200 pg of total WEHI cell RNA, but receptor signal can be detected from 2 ng of total bone marrow RNA.

EXAMPLE 2

Effect of Leptin on Proliferation of Hematopoietic Cells

This Example demonstrates that Leptin has a mitogenic effect on hematopoietic cells.

Hematopoiesis is regulated by a group of proteins known as hematopoietic growth factors and the constant presence of these factors is required for the proliferation and differentiation of hematopoietic cells. The effect of hematopoietic growth factors is mediated through the specific cell surface proteins known as hematopoietic growth factor receptors. In Example I the presence of leptin receptor sequence was detected in the bone marrow cDNA library. To explore the possible regulatory effect of leptin on hematopoiesis, the mitogenic effect of leptin on hematopoietic cells was assessed. In an initial experiment illustrated in Table 1, unfractionated bone marrow cells were plated in agar at a concentration of 5000 cells per dish under three different growth conditions: (1) medium alone, (2) leptin (added to medium), or (3) IL-3 (added to medium). Mouse IL-3 was obtained from Dr. A. Hapel, Australian National University (the activity of IL-3 was 5 U per 1 ng). As shown in Table 1,

no colony growth occurred in medium alone, 34±5 colonies grew in the presence of IL-3, and some clusters were noticed in plates with bone marrow cells grown in the presence of leptin. These results suggest that either the leptin effect on bone marrow cells is not significant or the target cell population is infrequent and the effect can not be seen clearly in non-fractionated marrow.

Table 1. Effect of leptin on the proliferation on unfractionated bone marrow cells

Growth Conditions	Number of Colonies per 5000 plated Cells	Number of Clusters per 5000 plated Cells
medium alone	0	0
IL-3 200 ng/ml	34±5	N.D.*
leptin 100 ng/ml	0	10-20

* N.D. = Not Determined, but <100.

The next series of experiments used different bone marrow fractions. Bone marrow was fractionated by depleting the marrow of cells that are committed to specific lineages using a cocktail of antibodies against cell surface lineage specific antigens. This "lin- fraction" is highly enriched for hematopoietic progenitor cells. Lineage committed cell depletion was performed as follows:

Three-to-six-month-old male BDF_1 mice (C57Bl/6 X DBA/2) were sacrificed, femurs and tibias were removed aseptically and receptively flushed of marrow with PBS with 2% FBS. Low density cells were first isolated on a gradient by layering 5-ml aliquots of 10^7 unfractionated cells/ml over 4.0 ml Nycodenze (1.080 g/ml) (Nycomed, Oslo, Norway). Cells were centrifuged 20 minutes at 400xg, collected from the interface and washed with a 10x excess of PBS with 2% FBS. Lineage committed cells were then removed by the antibody mediated magnetic bead depletion using the following rat anti-mouse

monoclonal antibodies: anti-7/4 (neutrophils and activated macrophages; obtained from S. Gordon, Oxford, UK), anti-YW25.12.7 (blasts and nucleated erythroid cells; obtained from I. Bertoncello, Peter MacCallum Cancer Institute, Melbourne, Australia). Antibodies purchased from PharMingen (San Diego, CA) were anti-Ter1-19 (nucleated erythroid cells), anti-B220 (B and pre-B lymphocytes), anti-Mac-1 (granulocytes, macrophages) and anti-Gr-1 (granulocytes, monocytes). Cells were resuspended in PBS with 2%FBS at a concentration $1x10^7$ cells/100μl of PBS and the equal volume of antibody mix was added (anti-YW 25.12.7 was added to the antibody mix at 10% of the volume). After an incubation for 15 minutes at 4°C, cells were washed by centrifugal sedimentation over a 2 ml FBS gradient to separate cells from unbound antibodies. Cells were then transferred into 50 ml centrifuge tubes (Corning) and rosetted with magnetic beads coated with sheep anti-rat IgG (Dynabeads M-450, Dynal, Inc., Great Neck, NJ) in the following manner: to an equal volume of cells ($350x10^6$ cells/ 3.5 ml maximum per tube), 10x the number of magnetic beads was added dropwise to the cell suspension and mixed gently with a pipette. The suspension was centrifuged at 20xg for 3 min, then vigorously resuspended and transferred to a 15 ml polypropylene tube. Then 4 ml of PBS with 10% was added to prevent beads trapping and a tube was placed into the Dynal magnet for 3 min. Cells not bound with the magnetic beads remained in suspension and were carefully removed with a pipette (this fraction was designated lineage negative cells, lin-). The lin- cells were counted and resuspended in 10mM Hoechst 33342 (Ho) (Sigma Chemical, St Louis, MO) in PBS with 10% FBS (adjusted to pH 7.2 with $NaHCO_3$) and incubated at 37°C for 1 hr. After 40 min of Ho incubation, 0.1 μg/ml of Rhodamine 123 (Rh 123) (Sigma) was added to the cell suspension, giving 20 min total incubation with Rh 123. After dye incubation, cells were centrifuged, chilled to 4°C, washed with PBS with 2% FBS and adjusted to $3x10^6$ cells per 100ml. Cells were then incubated for 15 minutes at 4°C with directly phycoerythrin-labeled anti c-kit antibody (2B8 clone,

PharMingen) at 1μg/ml, then washed and resuspended in PBS with 2% FBS. Finally, 2μg/ml of propidium iodide (Sigma) was added to the cell suspension for detection of dead cells, just prior to cell sorting.

At the next step of fractionation, lin- cells were selected based on their expression of c-kit on the cell surface into c-kit+ and c-kit- fractions. Previous data showed that the c-kit+ fraction is highly responsive to hematopoietic growth factors. To select for more primitive hematopoietic cell fraction, lin-/c-kit+ cells were separated based on the expression of different amount of Rhodamine 123 fluorescence into Rh low and Rh high fractions.

The pre-fractionated cells were analyzed and sorted on a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) equipped with dual argon lasers, and an automated cell delivery unit (ACDU). Cells were kept chilled at 4°C with a recirculating water bath. Monochromatic light at 351-364 nm and 488 nm was used for Ho and Rh 123 excitation, respectively. Forward light scatter was detected using 488bp10 and ND 1.0 filters. Rhodamine 123 emission was detected using a 530bp20 filter, PE emission using a 575bp20 filter, propidium iodide emission using a 610 lp filter, and Hoechst emission using 395bp20, 485bp15, or a 515 lp filter, as indicated.

The lin-/Rh low/c-kit+ fraction contains primitive hematopoietic cells, whereas cells in the Rh high fraction are less primitive. The different bone marrow fractions were then expose to leptin. In experiments illustrated in Table 2, the single cell culture system was used to determine the direct effect on the target cell population and to measure the cloning efficiency (the proportion of dividing cells).

In the single cell culture system, single sorted cell were directly deposited into 96-well U-bottomed plates (Corning) into Iscove's modified Dulbecco's medium (IMDM) (Gibco-BRL, Grand Island, NY) supplemented with cytokines as indicated and with 12.5% FBS, 12.5% horse serum (HS) (Gibco), $2x10^{-5}$mol/L 2-mercaptoethanol (2ME) (Sigma), 10^{-7} mol/L

hydrocortisone (HC) (Sigma), and antibiotics (penicillin/ streptomycin) (Gibco). Growth factors were used at the following concentrations: 50 ng/ml of SCF, 20 ng/ml of IL-6, 100 ng/ml of IL-3 in triple cytokine combination and 400 ng/ml of IL-3 used as a single growth factor. In all experiments, cells were cultured for 2-3 weeks. The use of U-bottom plates facilitated the settling of single cells to the bottom center, which allowed direct observation. Clones ranging from 2-64 cells could be directly enumerated. The number of cells per well were directly counted using a phase contrast microscope at 200 x magnification. Where indicated, single cell survival was determined by trypan blue exclusion coupled with the ability of live cells to highly refract light. Cell differentials were performed by counting cytospin preparations on slides stained by the Giemsa-Wright method.

Table 2 shows that leptin had a direct effect on cells from both the lin-/c-kit+ and lin-/Rh high/c-kit+, bone marrow fractions enriched for progenitor cells, where a substantial proportion of cells (20±5% and 22±6% of cells, respectively) proliferated in response to leptin. After 7 days of culture in leptin (100 ng/ml), the proliferating clones reached the size of 100-200 cells per clone, corresponding to 7-8 cell divisions.

In contrast, lin-/Rh low/c-kit+ bone marrow fraction that contained more primitive hematopoietic cells did not respond to leptin. These results showed that leptin has direct mitogenic on specific bone marrow cell populations.

Table 2: Effect of leptin on the proliferation of single BM cells

Bone Marrow Fraction	Cloning Efficiency in medium alone	Clone Size in medium alone at day 7	Cloning Efficiency in Leptin 200 ng/ml	Clone Size in Leptin 200 ng/ml at day 7
lin-/c-kit+ *	2±2	0	20±5	80±65
lin-/Rh low /c-kit+	0±0	0	0	0
lin-/Rh high /c-kit+	1±1	0	22±6	97±57

* no cell proliferation seen in lin-/c-kit- fraction

To further determine the effect of leptin on the proliferation of bone marrow cells, the rate of cell proliferation was analyzed in multiple cells per well. The above data showed that lin-/c-kit + bone marrow fraction contains cells that are direct target cells for leptin. To determine whether there are other target cells in this cell fraction that produce and release growth factors in response to leptin, lin-/c-kit+ cells were grown in the presence of leptin at different cell concentrations for seven days and the cell number then counted. The indicated number of lin- or lin-/c-kit+ cells was directly deposited from the sorter into 96 U-bottomed culture plates containing medium alone or medium with 200 ng/ml leptin. As shown in the Table 3, no proliferation of cells was observed in medium alone. The number of cells after seven days of culture in leptin increased proportionally with the number of cells per well plated at day 0. For example: 50 cells plated at day 0 grew into 440 cells after 7 days, indicating that the cell number expanded during the culture 8.8 fold compared to the cell number at the beginning of the culture. There was no significant difference in the rate of leptin induced cell proliferation between wells containing different cell number at the beginning of culture. Therefore, it appeared that leptin did not have an indirect effect on cell proliferation with lin-/c-kit+ cells.

Table 3: Effect of leptin on the proliferation of BM cells in multiple cells/well cell cultures

Bone Marrow Fraction	Number of Cells/well at day 0	Number of Cells/well after 7 days in medium alone	Number of Cells/well after 7 days in leptin 200 ng/ml	Increase in Cell Number after 7 days in leptin 200 ng/ml
lin-/c-kit+	50	0	440	8.8 fold
lin-/c-kit+	250	0	2250	9.0 fold
lin-/c-kit+	500	0	4000	8.0 fold
lin-/c-kit+	1000	0	12500	12.5 fold

In contrast, in the lin- cell population leptin had an indirect effect on cell proliferation. As shown in Table 4, the rate of the proliferative response to leptin increased significantly with increased number of lin- cells plated in the presence of leptin at day 0. This suggests that leptin induces the production of other hematopoietic growth factor(s) that stimulate proliferation on lin- cells.

Table 4: Effect of leptin on the proliferation of BM cells in multiple cells/well cell cultures (data from one rep. experiment).

Bone Marrow Fraction	Number of Cells/well at day 0	Number of Cells/well after 7 days in medium alone	Number of Cells/well after 7 days in leptin 200 ng/ml	Increase in Cell Number after 7 days in leptin 200 ng/ml
lin-	20	0	140±96	7 fold
lin-	40	1.7±2.8	182±102	4.6 fold
lin-	80	2.3±3.2	1407±379	17.5 fold
lin-	100	4±2	2213±533	22.1 fold
lin-	200	7±6	13333±6292	66.7 fold
lin-	400	25±9	308333±18764	77.1 fold

As hematopoietic progenitor cells give rise into functionally mature and differentiated blood cells, their proliferation and differentiation is dependent on the presence

of specific hematopoietic growth factors. In vitro, the number of progenitor cells can be assessed by colony formation in agar in the presence of hematopoietic growth factors. Thus, the effect of leptin on the generation of hematopoietic progenitor cells was determined. Different bone marrow fractions were grown in the presence of medium alone or leptin for 7 days at the different cell concentration. After this time, all the cells were transferred into the agar cultures containing IL-3, IL-6, and SCF and the number of progenitor cells was determined after additional 14 days of culture. Human IL-6 (rhuIL-6) was obtained from Immunex Corp., Seattle, WA, and rat SCF was obtained from Amgen Inc., Thousand Oaks, CA. Table 5 shows the number of progenitor cells derived from different bone marrow fractions in the presence of leptin. No progenitors were generated from lin-/c-kit- fraction, and no progenitors were seen in any of tested fractions after 7 days culture in medium alone. Thus, it appears that the number of progenitors detected in the presence of leptin was dependent on the number of lin- and lin-/Rh high/c-kit+ plated. In both of these fractions, progenitor cells were generated when 250 cells were put in the culture with leptin at day 0. In contrast, in the lin-/c-kit+ fraction the generation of progenitors could be measured when as few as 50 cells were cultured in the presence of leptin. These results indicate that leptin has an effect on the generation of hematopoietic progenitor cells, either on the self replication of progenitors cells and/or the generation of progenitor cells from less mature pre-progenitor cells.

Table 5: Effect of leptin on the generation of progenitor cells

Bone Marrow Fraction	% lin- Fraction	Number of Cells Cells at day 0	Number of Proginators at day 0	Number of Progenitors after 7 days in medium alone	Number of Progenitors after 7 days in leptin 200 ng/ml	Fold Change in Progen-tors Number
lin-	100	50	3*	0±0	4±1	1.3
		250	15	0±0	212±98	14.1
		500	30	0±0	325±31	10.8
		1000	60	0±0	480±149	8
			0	0	0	
lin-/c-kit-	50	50				
		250	0	0	0	
		500	0	0	0	
		1000	0	0	0	
lin-/c-kit+	50	50	5±1.5	0	90	18.0
		250	30±3	0	204	6.8
		500	60±6	0	604	10.1
		1000	120±12	0	1613	13.4
lin-/Rh low/c-kit+	5	50	36±11	1	5	0.14
		250	180±55	1	7	0.04
lin-/Rh High/c-kit+	20	50	9±4	0	0	0
		250	45±20	0	354	7.9

* number based on lin-/c-kit- + lin-/c-kit+

EXAMPLE 3

Leptin Supports Survival of a Hematopoietic Stem Cell Fraction Without Proliferation or Differentiation

In studies involving hematopoietic stem cells a significant problem is manipulating stem cells *in vitro* without the cells losing their viability and stemness. The presence of most of the known hematopoietic growth factors induces a proliferation of stem cells that is coupled with cell differentiation. The following experiments demonstrate that leptin can maintain hematopoietic stem cells without inducing proliferation or differentiation.

In experiments summarized in Table 6, single stem cells were cultured in the presence of medium alone or leptin at two different concentrations for 7 days. Single lin-/lowRh/lowHo/c-kit+ cells were directly deposited from the sorter into 96-well U-bottomed plates containing medium alone or medium with 100 ng/ml or 1 pg/ml leptin. The presence of cells in the well was verified by microscopic examination. After seven days cell viability was estimated by the exclusion of trypan blue and/or by the ability to proliferate in response to growth factors (50 ng/ml IL-3, 50 ng/ml SCF, and 20 ng/ml IL-6). Data represent mean values ± SD from 3-7 independent experiments, where 80-90 single cells were analyzed. These results indicate that leptin acts as surviving factor for a subpopulation of stem cells.

Table 6: Effect of leptin on surviving of single lin-/lowRh/lowHo/c-kit+ cells

% single cells after 7 days in medium alone	% single cells after 7 days in leptin 100 ng/ml	% single cells at after 7 days in leptin 1 pg/ml
1.2±0.9	13±9	15±10

To further study the effect of leptin on maintenance of stem cells *in vitro*, cells from different bone marrow fractions were grown in the presence of leptin for 7 days and their proliferative potential then assayed in agar. For HPP-CFC and GM-CFC assays, a double-layer nutrient agar culture was used consisting of a 1 ml underlayer of 0.5% agar plus hematopoietic growth factors: rrSCF (50ng/ml), rmIL-3 (115ng/ml), rhIL-1 (20ng/ml), rhCSF-1 (500U/ml) and 0.5 ml overlay of 0.3% agar plus target cells in 35-mm Petri dishes was used. Cultures were incubated at 37°C in a 5% O_2, 5% CO_2, 90% N_2 gas mixture. HPP-CFC and GM-CFC were enumerated at 14 days using a dissecting microscope at 10X magnification or an inverted microscope at 25X magnification, respectively. HPP colonies were identified as macroscopic colonies (>50,000 cells/colony) with diameters >1.0 mm with a dense center.

The results, shown in Table 7, confirm the results above that a subpopulation of stem cells present in lin-/Rh low/c-kit+ fraction is able to survive in leptin (added to medium) for 7 days and retain their proliferative potential. Previous experiments showed that after 7 days culture in other growth factor combinations stem cells either did not survive or differentiated (no HPP formation detected). The results of this experiments are striking because stem cells were the only cell population that survived for 7 days in leptin in lin-/Rh low/c-kit+ fraction.

Table 7

Bone Marrow Fraction	Number of Cells Plated	Number of HPP-CFC plated at day 0	Number of cells plated at day 0 in leptin 200 ng/ml	Number of HPP-CFC after 7 days in leptin 200 ng/ml	Number of Clusters after 7 days in leptin 200 ng/ml
lin-/c-kit+	250	27	250	0	204
lin-/c-kit-	250	0	250	0	0
lin-/Rh low /c-kit+	250	159	250	4	3
lin-/Rh high/c-kit+	250	43	250	0	354

EXAMPLE 4

Leptin Inhibits Cell Division In a Sub-population of IL-3 Responsive Hematopoietic Cells

The effect of leptin on the response of bone marrow cells to other hematopoietic growth factors was determined. IL-3 is an important growth factor for a wide variety of bone marrow cells that are at the different stages of hematopoietic cell differentiation. The results demonstrate that leptin has an inhibitory effect on colony formation that occurs in different bone marrow fractions in response to IL-3.

In the experiment shown in Table 8, different bone marrow fractions were grown in agar in the presence of IL-3 and different concentrations of leptin. After 14 days of culture, the number of colonies was estimated. The results (Table 8) showed that leptin significantly inhibited the growth of a population of IL-3 responsive hematopoietic cells that were present in unfractionated bone marrow and in different purified fractions. This inhibition appeared to be dose dependent and was detected within physiological concentrations of leptin.

Table 8: Effect of leptin on the colony formation in response to IL-3

Growth Conditions	Unfractionated BM Number of Colonies/5000 Cells	% of Inhibition	Post Gradient BM Number of Colonies/5000 Cells	% of Inhibition	Lineage depleted BM Number of Colonies/5000 Cells	% of Inhibition
IL-3	17±3		30±4		280±3	
IL-3 +Leptin 1 μg	15±3	12	26±4	13	220±3	21
IL-3 +Leptin 100 ng	10±3	41	16±2	47	150±4	46
IL-3 +Leptin 10 ng	14±4	18	33±3	0	240±4	14
IL-3 +Leptin 100 pg	17±4	0	31±4	0	280±2	0
IL-3 +Leptin 1 pg	15±3	0	33±2	0	290±2	0
IL-3 +Leptin 10 fg	18±6	0	28±5	0	300±5	0
IL-3 +Leptin 0.1 fg	18±1	0	27±5	0	290±5	0

The observation that leptin inhibits a proliferation of substantial fraction of IL-3 responsive cells led to determining the effect of leptin on the proliferation of individually cultured stem cells in response to IL-3. Previous data showed that IL-3 responding cells represent short term repopulating stem cell population (STR-HSC). As shown in Table 9, in three independent experiments the IL-3 stimulated cell proliferation was significantly inhibited by leptin. This inhibitory effect was direct, as observed in single cell cultures.

Table 9: Effect of leptin on the proliferation of hematopoietic stem cells in response to IL-3

Experiment #	Cloning Efficiency IL-3	Cloning Efficiency IL-3 + leptin 100 ng/ml
1	45%	29%
2	19%	10%
3	10%	4% (leptin 200 ng/ml)

In summary, the foregoing Examples clearly show that leptin affects hematopoiesis in different ways and can act on cells at different stages of differentiation. Starting at the top of the bone marrow cell hierarchy, leptin acts as a surviving factor for a subpopulation of primitive hematopoietic stem cells. In the presence of leptin the generation of progenitor cells occurs. Leptin also inhibits the proliferative response to IL-3 in wide variety of bone marrow cells, and leptin acts as direct mitogen for a subpopulation of lin-/c-kit+ cells.

While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be

determined with reference to the appended claims along with their full scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: USE OF LEPTIN TO STIMULATE HEMATOPOIESIS

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(v) CURRENT APPLICATION DATA:
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(B) FILING DATE: 25-OCT-96

(vii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: 17881-1

(viii) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (206) 467-9600
(B) TELEFAX: (415) 576-0300

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGTGGTTTTG TTACAYTGGG 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGTGAYTTCC ATATGCAATC C 21

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACACTGTTAA TTTCACACCA GAG 23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGTCATTCAA ACCATTAGTT TAGG 24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACACTGTTAA TTTCACACCA GAG 23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGGATAAACC CTTGCTCTTC A 21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACACTGTTAA TTTCACACCA GAG 23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGAACACAAC AACATAAAGC CC 22

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACACTGTTAA TTTCACACCA GAG 23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGGCTCCCTC AGGGCCAC 18

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGTTATATCT GGTTATTGAA TGG 23

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CATTAAATGA TTTATTATCA GAATTGC 27

WHAT IS CLAIMED IS:

1. A method for increasing the number of hematopoietic precursor cells, comprising:

exposing the hematopoietic precursor cells to leptin, thereby increasing the number of precursor cells relative to the number of cells initially present.

2. The method of claim 1, wherein the hematopoietic precursor cells are separated from mature hematopoietic cells present in a blood product prior to exposing the hematopoietic precursor cells to leptin.

3. The method of claim 1, wherein the hematopoietic precursor cells are human.

4. The method of claim 1, wherein the hematopoietic precursor cells are hematopoietic stem cells.

5. The method of claim 1, wherein the hematopoietic precursor cells are hematopoietic progenitor cells.

6. The method of claim 1, wherein the hematopoietic precursor cells are lin- cells.

7. The method of claim 6, wherein the hematopoietic precursor cells are lin-/c-kit+ cells.

8. The method of claim 7, wherein the hematopoietic precursor cells are lin-/Rh low/c-kit+ cells.

9. The method of claim 7, wherein the hematopoietic precursor cells are lin-/Rh high/c-kit+ cells.

10. The method of claim 2 wherein the blood product is bone marrow, umbilical cord blood, or peripheral blood.

11. The method of claim 1, wherein the hematopoietic precursor cells are exposed to the leptin *in vitro*.

12. The method of claim 1, wherein the hematopoietic precursor cells are exposed to the leptin *in vivo*.

13. The method of claim 11, wherein the hematopoietic precursor cells are exposed to the leptin *in vitro* in a culture medium including at least one exogenously added growth factor.

14. The method of claim 13, wherein the growth factor is G-CSF, GM-CSF, SCF, IL-1, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 or TGF-β.

15. A method for maintaining the number of hematopoietic stem cells *in vitro*, comprising:

exposing the hematopoietic stem cells to leptin and culturing the cells, thereby maintaining the number of stem cells relative to the number of cells initially present.

16. The method of claim 15, wherein the hematopoietic stem cells are separated from mature hematopoietic cells present in a blood product prior to exposing the hematopoietic stem cells to leptin.

17. The method of claim 16, wherein the hematopoietic stem cells are human.

18. The method of claim 17, wherein the hematopoietic stem cells are lin-/c-kit+ cells.

19. The method of claim 16 wherein the blood product is bone marrow or umbilical cord blood.

20. A method for sustaining primitive hematopoietic stem cells *in vitro* without proliferation or differentiation, comprising:

exposing the primitive hematopoietic stem cells to leptin and culturing the cells, thereby sustaining the number of primitive stem cells relative to the number of cells initially present.

21. The method of claim 20, wherein the primitive hematopoietic stem cells are lin-/Rh low/Ho low/c-kit+ or lin-/c-kit+.

22. The method of claim 20, wherein the primitive hematopoietic stem cells are separated from mature hematopoietic cells present in a blood product prior to exposing the primitive hematopoietic stem cells to leptin.

23. The method of claim 20, wherein the primitive hematopoietic stem cells are sustained for at least five days.

24. The method of claim 20, wherein the primitive hematopoietic stem cells are subsequently stimulated to proliferate and differentiate.

25. A method for inhibiting proliferation of hematopoietic cells stimulated by IL-3, comprising:

exposing the IL-3 stimulated hematopoietic cells to leptin, thereby inhibiting the proliferation of cells induced by IL-3.

26. The method of claim 25, wherein the hematopoietic cells are in unfractionated bone marrow.

27. The method of claim 25, wherein the hematopoietic cells are precursors separated from mature hematopoietic cells present in a blood product prior to exposing the hematopoietic precursor cells to leptin.

28. A method for increasing the number of hematopoietic cells in a mammalian host in need thereof, comprising:

exposing the hematopoietic precursor cells of the host to leptin, thereby increasing the number of precursor cells relative to the number of cells initially present.

29. The method of claim 28, wherein the hematopoietic precursor cells are exposed to leptin *in vitro*.

30. The method of claim 29, wherein the hematopoietic precursor cells are separated from mature hematopoietic cells present in a blood product prior to exposing the hematopoietic precursor cells to leptin.

31. The method of claim 30, wherein the hematopoietic precursor cells are obtained from the host prior to initiation of cytotoxic therapy.

32. The method of claim 30, wherein the hematopoietic precursor cells are lin- cells.

33. The method of claim 32, wherein the hematopoietic precursor cells are lin-/c-kit+ cells.

34. The method of claim 30, wherein the blood product is bone marrow.

35. The method of claim 28, wherein leptin is administered to the mammalian host and the hematopoietic cells are exposed *in vivo*.

36. The method of claim 35, wherein the leptin is administered intravenously or intramuscularly.

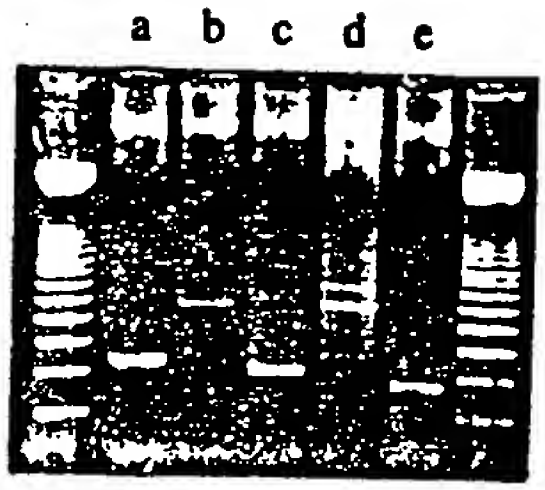
37. A composition for sustaining or expanding the number of hematopoietic precursor cells which comprises a nutrient medium and leptin.

38. The composition of claim 37, further comprising at least one exogenously supplied growth factor.

39. The composition of claim 38, wherein the growth factor is granulocyte colony stimulating factor or stem cell factor.

Fig. 1

Fig. 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18875

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : A61K 39/00, 38/18, 38/00, C07K 14/00
US CL : 424/198.1, 85.1; 514/12; 530/399
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/198.1, 85.1; 514/12; 530/399

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, REGISTRY, CA, BIOSIS, BIOTECHDSMEDLINE, EMBASE, LIFESCI, WPIDS, CONFSCI, DISSABS, SCISEARCH
search terms: leptin receptor, leptin, hematopoiesis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	BENNETT et al. A role for leptin and its cognate receptor in hematopoiesis. Current Biology. 01 September, 1996. Vol 6. No. 9 pages 1170-1180, especially Abstract.	1-5, 10-12 ---------- 6-9, 13-39
Y	PARK et al. Primitive human hematopoietic precursors express Bcl-x but not Bcl-2. Blood. 01 August 1995. Vol 86. No. 3. pages 868-876, see entire article.	6-9, 13-39

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 DECEMBER 1997	Date of mailing of the international search report 03 FEB 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer [illegible] Telephone No. (703) 308-[illegible]

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